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Genetic Relationships Among Salamanders of the Genus *Hynobius* (Amphibia, Caudata) from Korea and Southwestern Japan

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We performed allozyme analysis for three Korean (*Hynobius leechii, H. quelpaertensis,* and *H. yangi*) and three Japanese (*H. nebulosus*, *H. tsuensis*, and *H. dunni*) salamanders to clarify their interspecific relationships using *H. naevius* as an outgroup. The genetic distances (Nei's D) within ingroup species ranged from 0.11 to 0.78 with a mean of 0.33. In the NJ and CONTML trees, monophyly of the ingroup was not supported and Korean *H. quelpaertensis* and *H. leechii* diverged first from the remaining species, which together formed a weakly supported clade. Korean *H. yangi*, long identified as *H. leechii*, was closer to Japanese *H. nebulosus* (D=0.108) and *H. tsuensis* (D=0.138) than to Korean *H. leechii* (D=0.197) and *H. quelpaertensis* (D=0.305). *Hynobius tsuensis* and *H. nebulosus* were very close (D=0.108) despite their different breeding habits. A geohistorical hypothesis is proposed to explain the divergence of the six species.

Key words: allozyme, biogeographic history, breeding habit, molecular clock, phylogenetic relationships

INTRODUCTION

Small salamanders of the genus *Hynobius* Tschudi, 1838 occur in East Asia and are differentiated into many species that are usually very similar in morphology. Sato (1943) roughly divided members of this genus from Japan, Korea, and Taiwan into three types, the *H. nebulosus* group adapted to lentic breeding in warm climatic conditions in the lowlands, the *H. lichenatus* group adapted to lentic breeding in cool climatic conditions in the lowlands, and the *H. naevius* group adapted to lotic breeding in cool climatic conditions in the mountains.

Sato (1943) placed six species in the *H. nebulosus* group: *H. leechii* Boulenger, 1887, *H. nebulosus* (Temminck and Schlegel, 1838), *H. tokyoensis* Tago, 1931, *H. dunni* Tago, 1931, *H. tsuensis* Abe, 1922, and *H. formosanus* Maki, 1922, in Korea, Japan, and Taiwan. The same author (Sato, 1943) placed *H. okiensis* Sato, 1940, *H. naevius* (Temminck and Schlegel, 1838), *H. kimurae* Dunn, 1923, and *H. stejnegeri* Dunn, 1923 from Japan, and *H. sonani* (Maki, 1922) from Taiwan, in the *H. naevius* group.

The phylogenetic relationships of *H. tokyoensis*, placed in the *H. nebulosus* group by Sato (1943), are now in dispute (*e.g.*, Matsui, 1987; Matsui *et al*., 2001, 2007a), and *H. tsuensis* and some populations of *H. nebulosus* are strictly not lentic, but lotic breeders (Matsui *et al*., 2006). Further, *H. formosanus* is now considered to be not a lentic, but a lotic breeder (Kakegawa *et al*., 1989), and *H. okiensis*, despite its lotic breeding habit, is estimated to be close to the *H. nebulosus* group (Matsui *et al*., 2007b).

Through recent genetic studies in Korea (*e.g.*, Yang *et al*., 1997; Kim *et al*., 2003), *H. quelpaertensis* Mori, 1928 and *H. yangi* Kim, Min, and Matsui, 2003 have been split from *H. leechii* as distinct species, and can be regarded as members of the *H. nebulosus* group of Sato (1943). However, close morphological similarities of the species in this group have hindered assessment of their phylogenetic relationships. In this study, we surveyed genetic relationships among three Korean and three Japanese salamanders of the *H. nebulosus* group to clarify their phylogenetic relationships.

MATERIALS AND METHODS

A total of 58 salamanders of three species from Korea (*H. leechii* [n=10, from Kyeonggi Pref.]; *H. quelpaertensis* [n=10, from Jeju (=Cheju) Isl., Jeju Pref.]; *H. yangi* [n=8, from Kijang-gun, Busan City]) and three species from Japan (*H. nebulosus* [n=11, from Isahaya-shi, Nagasaki Pref.]; *H. tsuensis* [n=10, from Tsushima Isl., Nagasaki Pref.]; *H. dunni* [n=9, from Bungotakada-shi, Oita Pref.]) were collected during 1990 and 2001 (Fig. 1). Additionally, six specimens of *H. naevius* from Unnnan-shi (former Daito-cho), Shimane Pref. Japan were used as an outgroup. Recent studies revealed the presence of distinct genetic groups in *H. nebulosus* (Matsui *et al*., 2006), and the population used here is from near the type locality. Also, two distinct taxa are present in *H. naevius*, and the population used here corresponds to the large-sized group (Group A) of Tominaga *et al*. (2005a, b). Samples of liver were removed and maintained frozen at –84°C until use for electrophoresis. Voucher specimens were fixed in 10% formalin and later preserved in 70%

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ethanol, and are stored at Kyoto University (KUHE). We analyzed homogenized tissue extracts by standard horizontal starch gel electrophoresis (Shaw and Prasad, 1970; Ayala *et al*., 1972) at a concentration of 11.5%. We scored the products of 22 loci encoding 15 allozymes for all individuals, as shown in Table 1.

Genetic interpretations of allozyme data were based on criteria developed by Selander *et al*. (1971). Enzyme nomenclature, E.C. number, and the notation of loci, electromorphs and genotypes mainly follow IUBMB (1992) and Murphy *et al*. (1996). We designated electromorphs by letters with 'a' representing the most slowly migrating variant. All statistics were calculated by using the BIOSYS-1 computer program (Swofford and Selander, 1981). Standard estimates of genetic variability, *i.e*., mean number of allele per locus (A), proportion of polymorphic loci (P), and mean het-

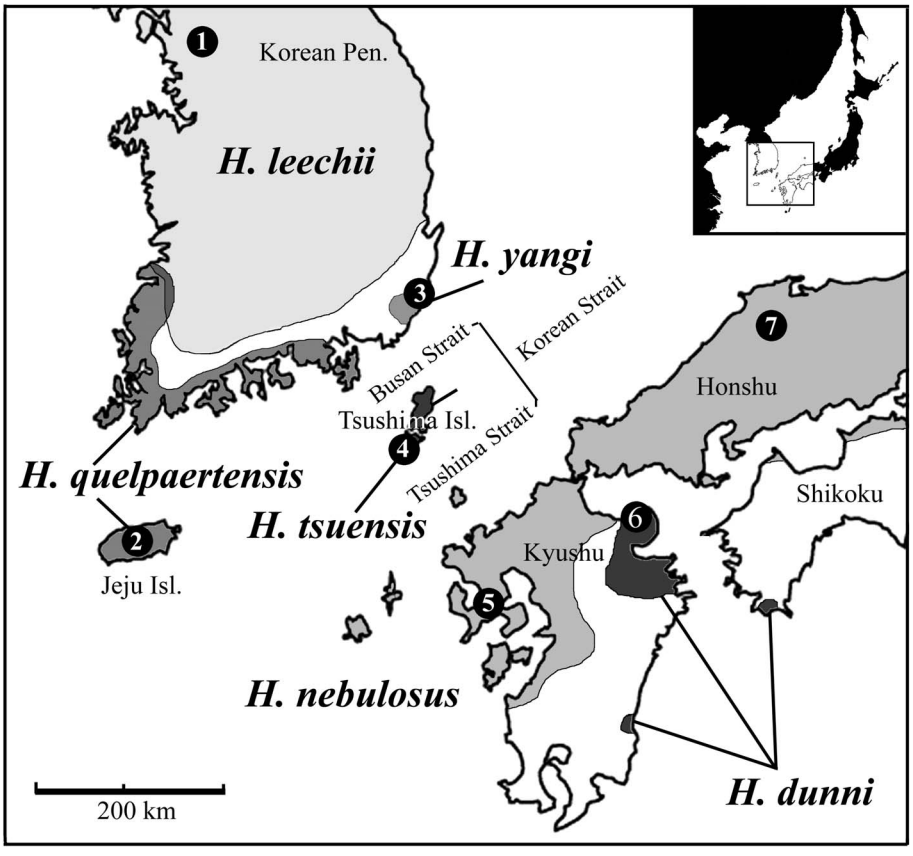

Fig. 1. Map of Korea and Japan, showing the sampling localities (1, *H. leechii*; 2, *H. quelpaertensis*; 3, *H. yangi*; 4, *H. tsuensis*; 5, *H. nebulosus*; 6, *H. dunni*; 7, *H. naevius*) and distributional ranges of the six ingroup species of *Hynobius* used in the electrophoretic analysis.

Table 1. Enzymes, loci and buffer systems used in the analyses of allozyme variation in *Hynobius* species.

Enzyme	E.C. number	Locus	Buffer system[a]
Aconitate hydratase	4.2.1.3	*Acon-1, 2*	TC8
Alcohol dehydrogenase	1.1.1.1	*Adh*	TBE8.7
Asparate transaminase	2.6.1.1	*Ata-1, 2*	CAPM6, TC7
Fumarate hydratase	4.2.1.2	*Fumh*	TBE8.7
Glucose-6-phosphate isomerase	5.3.1.9	*Gpi*	CAPM6
Glutamate dehydrogenase	1.4.1.3	*Gtdh*	TC8
Glycerol-3-phosphate dehydrogenase	1.1.1.8	*G3pdh*	TC8
Isocitrate dehydrogenase	1.1.1.42	*Idh-1, 2*	TC7
L-Lactate dehydrogenase	1.1.1.27	*Ldh-1, 2*	CAPM6, TC7
Malate dehydrogenase	1.1.1.37	*Mdh-1, 2*	CAPM6, TC8
Malic enzyme[b]	1.1.1.40	*Mdhp-1, 2*	TC7
Peptidase (leucyl-alanine)	3.4.11.–	*Pep-la*	TBE8.7
Phosphogluconate dehydrogenase	1.1.1.44	*Pgdh*	TC7
Phosphoglucomutase	5.4.2.2	*Pgm-1, 3*	TC7
Superoxide dismutase	1.15.1.1	*Sod*	TBE8.7

[a]Buffer systems--CAPM6: Citrate-aminopropylmorpholine, pH=6.0 (Clayton and Tretiak, 1972), TC7: Tris-citrate, pH=7.0 (Shaw and Prasad, 1970), TC8: Tris-citrate, pH=8.0 (Clayton and Tretiak, 1972), TBE8.7: Tris-borate-EDTA, pH=8.7 (Boyer *et al*., 1963).

[b]NADP-dependent malate dehydrogenase.

erozygosity by direct count (H), were computed for each species.

We calculated coefficients of Nei's genetic distance (Nei, 1972), to facilitate comparisons with previous studies (Yang *et al*., 1997), and Cavalli-Sforza and Edwards' (1967) chord distance among species. To estimate genetic relationships among species, we performed a neighbor-joining (NJ) analysis (Saitou and Nei, 1987) using Cavalli-Sforza and Edwards' (1967) chord distance, and a continuous maximum-likelihood (CONTML: Felsenstein, 1973) analysis based on allelic frequencies. We designated *H. naevius* as an outgroup species. We assessed the validity of topologies obtained using 1,000 bootstrapping pseudo-replicates (Felsenstein, 1985). These analyses were performed by use of PHYLIP vers. 3.5 C (Felsenstein, 1993).

RESULTS

Of the 22 presumptive loci scored, three (*G3pdh, Gtdh,* and *Pep-la*) were fixed identically in all samples, and the remaining 19 loci were polymorphic; the most variable loci were *Ata-2*, *Gpi*, and *Ldh-2*, each with five alleles (Table 2). Among the ingroup species, the mean number of electromorphs per locus (A) varied from 1.2 in *H. dunni* to 1.6 in *H. leechii* and *H. yangi*, the percentage polymorphic loci (P) from 18.2 in *H. dunni* to 45.5 in *H. leechii*, and the mean heterozygosity by direct count (H) from 0.024 in *H. dunni* to 0.116 in *H. nebulosus* (Table 2). In the outgroup species, *H.*

Table 2. Allelic frequencies and genetic variability (1SE) at 19 polymorphic loci among seven *Hynobius* species. A=mean number of alleles per locus; P=percentage of loci; H=mean heterozygosity (direct count).

	Species						
Locus	*H. leechii*	*H. quelpaertensis*	*H. yangi*	*H. tsuensis*	*H. nebulosus*	*H. dunni*	*H. naevius*
Acon-1	a(0.050) b(0.900) c(0.050)	a	b	a(0.750) b(0.250)	b(0.818) c(0.182)	b	a
Acon-2	b(0.050) c(0.950)	c	b(0.875) c(0.125)	b	b(0.500) c(0.500)	b	a
Adh	d	c(0.100) d(0.900)	b(0.188) c(0.562) d(0.250)	c	c	b	a
Ata-1	a	b	a	a	a	a	a
Ata-2	b(0.100) c(0.800) d(0.100)	b(0.300) c(0.650) e(0.050)	c	a(0.150) c(0.850)	c(0.864) e(0.136)	a	c
Fumh	b	b	b	b	b	a(0.050) b(0.950)	b
Gpi	b(0.650) c(0.350)	a(0.050) b(0.950)	a(0.063) b(0.874) c(0.063)	a(0.200) b(0.650) c(0.150)	b(0.182) c(0.727) d(0.091)	c	a(0.167) e(0.833)
Idh-1	a	a	a	a	a(0.955) b(0.045)	a	a
Idh-2	c	c	c	b	b	a	d
Ldh-1	b	c	b	b	b	b	a
Ldh-2	a(0.050) b(0.950)	d	c	c	c(0.909) d(0.091)	c	b(0.833) e(0.167)
Mdh-1	b	b	b	b	b	a	b
Mdh-2	d	b(0.050) c(0.950)	a(0.124) c(0.813) d(0.063)	a(0.050) c(0.950)	c	b(0.056) c(0.944)	a
Mdhp-1	b(0.150) c(0.800) d(0.050)	b(0.600) c(0.300) d(0.100)	b(0.625) c(0.125) d(0.250)	c	b(0.955) c(0.045)	a	a
Mdhp-2	a(0.950) b(0.050)	a	a	a	a	a	a
Pgdh	a(0.050) c(0.950)	c(0.950) d(0.050)	b(0.124) c(0.813) d(0.063)	a(0.100) c(0.900)	c(0.955) d(0.045)	b(0.111) c(0.889)	c
Pgm-1	a(0.950) b(0.050)	a	a	a	a	a	a
Pgm-3	b(0.300) c(0.600) d(0.100)	a(0.050) c(0.950)	b(0.250) c(0.750)	b(0.100) c(0.700) d(0.200)	b(0.227) c(0.773)	b(0.050) c(0.950)	b
Sod	b	b	a(0.124) b(0.688) c(0.188)	b	b(0.818) c(0.182)	c	b
A	1.6 (0.2)	1.4 (0.1)	1.6 (0.2)	1.4 (0.1)	1.5 (0.1)	1.2 (0.1)	1.1 (0.1)
P	45.5	31.8	36.4	27.3	31.8	18.2	9.1
H	0.091 (0.029)	0.050 (0.024)	0.114 (0.036)	0.068 (0.034)	0.116 (0.033)	0.024 (0.012)	0.015 (0.015)

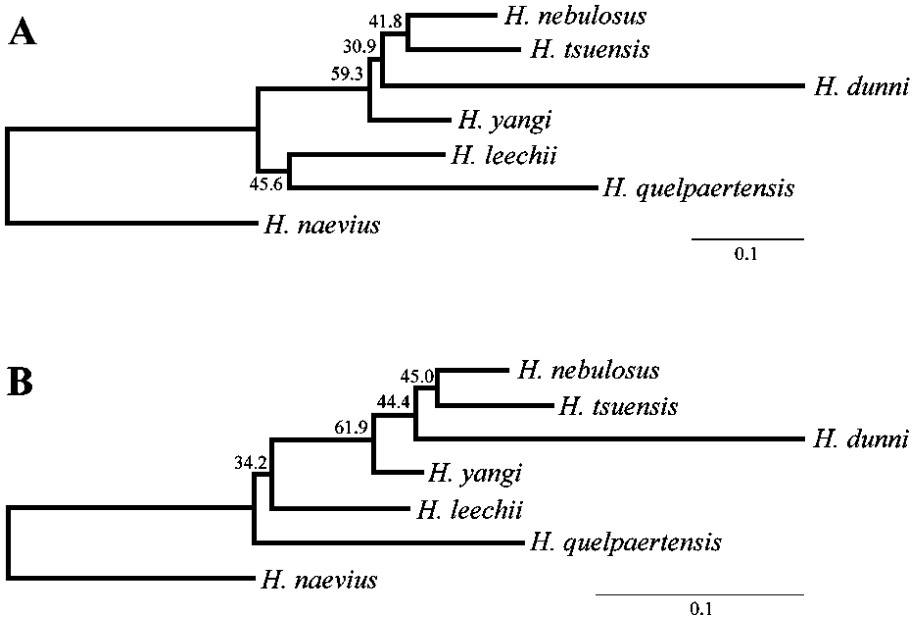

Fig 2. **(A)** NJ tree based on Cavalli-Sforza and Edwards' (1967) chord distance. **(B)** CONTML tree. Both trees are rooted by the outgroup *H. naevius*. Nodal values in the trees indicate percent support for branches in 1,000 bootstrap replicates.

naevius, A, P, and H were less variable than in the ingroup species (A=1.1, P=9.1, H=0.015).

Based on allelic frequencies listed in Table 2, genetic distances between each two species were calculated (Table 3). Nei's (1972) D values between the six ingroup species ranged from 0.108 (*H. nebulosus* vs. *H. tsuensis* and *H. yangi*) to 0.781 (*H. quelpaertensis* vs. *H. dunni*) with an average of 0.334. The genetic distance (D=0.108) between *H. nebulosus* and both *H. yangi* and *H. tsuensis* was surprisingly low, but there was one fixed difference at *Idh-2* from *H. yangi*. Similarly, a diagnostic difference (at the 95% confidence level: Ayala *et al*., 1972) at *Mdhp-1* was found between *H. nebulosus* and *H. tsuensis*, although they had no fixed allelic difference.

In the NJ and CONTML trees rooted by *H. naevius* (Fig. 2), monophyly of the ingroup species was not supported. The topology of the two trees was identical except for the relationships of two basal species, *H. leechii* and *H. quelpaertensis* (Fig. 2). Thus, the three Korean species were not a nested set of sister species in the two trees. Instead, *H. yangi*, long identified as *H. leechii* (Kim *et al*., 2003), formed a clade with three Japanese species. Although this clade was weakly supported in both trees (59.3% in NJ and 61.9% in CONTML), it contained *H. dunni*, which was the most distant (mean Nei's [1972] D=0.486) among six ingroup species, and the relationships of the four species nested were not resolved.

Table 3. Cavalli-Sforza and Edwards' (1967) chord distance (above diagonal) and Nei's (1972) genetic distance (below diagonal) among seven species of the genus *Hynobius* from Korea and Japan.

Species	1	2	3	4	5	6	7
1. *H. leechii*	–	0.412	0.263	0.408	0.380	0.692	0.620
2. *H. quelpaertensis*	0.305	–	0.415	0.506	0.498	0.884	0.737
3. *H. yangi*	0.197	0.320	–	0.222	0.170	0.449	0.677
4. *H. tsuensis*	0.316	0.388	0.138	–	0.180	0.511	0.621
5. *H. nebulosus*	0.293	0.398	0.108	0.108	–	0.462	0.708
6. *H. dunni*	0.575	0.781	0.331	0.406	0.339	–	0.863
7. *H. naevius*	0.473	0.589	0.528	0.493	0.547	0.776	–

DISCUSSION

The genetic distances obtained among the salamander species were not always concordant with the pattern of their geographic distribution. For example, Nei's (1972) D=0.197 obtained between *H. leechii* and *H. yangi (*Table 3), which are nearly parapatric on the Korean Peninsula (Fig. 1), was larger than the distance between Korean *H. yangi* and Japanese *H. nebulosus* (D=0.108) which are separated by the Korean Strait (=Busan and Tsushima Straits). This discrepancy must be derived from the history of divergence in these salamanders, in relation to the geohistry of the region connecting the Asian continent and Japan.

Although the accurate time of divergence in the salamanders treated here is unknown, some geological data allow us to estimate the history of their divergences. Because salamanders are considered to have poor migratory abilities (Sato, 1943), and also cannot cross over the sea, a strait must be a sufficient barrier to prevent gene flow between populations.

The main islands of Japan, including Kyushu, and Tsushima Isl. were connected to the continent through the Korean Peninsula before the formation of the straits and during later glaciations (Oshima, 1990; Ota *et al*., 2002; Park *et al*., 1996). Thus, the biogeography of the salamanders treated here was probably strongly influenced by past fluctuations in sea level between today's Korea and Kyushu. According to recent geological information, the Korean (Tsushima) Strait formed first around 1.7 Ma (Kitamura and Ubukata, 2003), long before the last formation of the strait between 0.15–0.07 Ma during the Riss-Wurm interglacial (Ohshima, 1990). Thus, Korean *H. yangi* and Japanese *H. nebulosus* and *H. tsuensis*, which are genetically very close in our results and are now separated

by the Korean Strait, may have differentiated following the first formation of the strait around 1.7 Ma.

On the other hand, Jeju Island, which is now the main habitat of *H. quelpaertensis*, is reported to have formed through four steps of volcanic activity that first occurred 1.2 Ma, and was separated from the Korean Peninsula by the formation of the Jeju Strait by 0.1 Ma (Park, 1988; Yang *et al*., 2000). Because small populations of *H. quelpaertensis* also occur in the southwestern and southern peripheral regions of the Korean Peninsula, both on small islands and mainland itself (Yang *et al*., 1997; Kim *et al*., 2003), the species is judged to be split by the formation of the Jeju Strait. From these lines of geological information, it is surmised that the final block of gene exchange between the Peninsula and Jeju Island occurred at about 0.1 Ma.

Nei's (1975) idea of a molecular clock, in which the genetic distance (D) estimated electrophoretically between taxa is proportional to their divergence time (T) from the common ancestor, might produce useful working hypotheses, if reliable temporal calibration is applied to available data. As shown above, the Nei's (1972) D between Korean *H. yangi* and Japanese *H. nebulosus* was 0.108, and constraining their separation at 1.7 Ma (Kitamura and Ubukata, 2003) results in the calibration of 1D=15.74 MY. For *H. quelpaertensis*, Yang *et al*. (1997) gave a minimal D of 0.007 between populations of Jeju Isl. and Jindo Isl. (situated at the periphery of the Peninsula). If we similarly constrain separation times of *H. quelpaertensis* between Jeju and Jindo at 0.1Ma (strait formation), the calibration of 1D results in 14.29 MY.

These calibrations (1D=14.29 and 15.74 MY) are surprisingly close to those reported for other urodeles. Hayashi and Matsui (1988) calibrated the molecular clock at 1D=13–22 MY in Japanese *Cynops*, which values include 1D=14 MY calibrated by Maxson and Maxson (1979) in plethodontid salamanders, and have generally been used in the study of salamanders (Larson *et al*., 1981; Larson, 1983; Matsui *et al*., 1992, 2000). Beerli *et al*. (1996) also computed slightly smaller estimates, 1D of 10–12.5 MY, in some European anurans. Accordingly we believe that using a calibration of 1D=14 MY is appropriate for urodeles.

Using the calibration of 1D=14 MY and Nei's D in Table 3, the history of divergences in the species treated here, in the area encompassing today's Korean Peninsula through Tsushima to southwestern Japan, is hypothesized in the following way. If an originally lentic breeding ancestor of the *H. nebulosus* group separated from a lotic breeding ancestor leading to *H. naevius*, the event seems to have occurred around 6.6–10.9 Ma in the late Miocene to early Pliocene. This estimation of ages does not contradict the time of divergence within *H. naevius* (sensu lato) around 2.9 Ma (Tominaga *et al*., 2006).

Within the *H. nebulosus* group, two lineages, one leading to *H. quelpaertensis* and *H. leechii* and another leading to the other species, diverged in the northern and southern areas, respectively, around 4.1–10.9 Ma in the late Miocene to middle Pliocene, although the common ancestry of *H. quelpaertensis* and *H. leechii* is not certain. In the northern lineage, the ancestral *H. quelpaertensis* and *H. leechii* would have diverged around 4.3 Ma in the middle Pliocene. After this separation, ancestral *H. quelpaertensis* on today's Peninsula would have narrowed its range towards the south, possibly through interspecific competition with the ancestor of *H. leechii*; with the formation of Jeju Island around 1.2 Ma, it moved its range farther south, and the new island became its main habitat. Some populations, however, were left behind as relicts in the peripheral regions of the Peninsula by the formation of Jeju Strait around 0.1 Ma in the late Pleistocene.

In the southern lineage, ancestral *H. dunni* first diverged allopatrically from the ancestors of the remaining species in the area of today's Kyushu, as long ago as around 4.6–5.7 Ma, in the middle Pliocene. The remaining ancestral stocks of the southern lineage also began allopatric divergence around 4.6 Ma, in the middle Pliocene, in three parts of the southeastern region of today's Korean Peninsula (*H. yangi*), Tsushima (*H. tsuensis*), and Kyushu (*H. nebulosus*). Finally, they would have been completely separated at 1.7 Ma, in the early Pleistocene, by the formation of the Korean Strait (=Busan and Tsushima Straits).

Hynobius leechii now occurs widely in inland Korea and neighboring China, whereas *H. yangi* is isolated in the Milyang sub-basin of the Kyeongsang Basin in the southeastern region. They are now separated by the Taehwa river, and this river system seems to have played an important role in blocking genetic interchange between the two species (Kim *et al*., 2003). It is possible that the ancestral *H. yangi,* after nearly simultaneous divergence from ancestors of *H. tsuensis* and *H. nebulosus*, could not further invade the interior of the Peninsula, where ancestral *H. leechii* already occurred.

Close genetic similarities of these three species were unexpected, because *H. nebulosus* shows high intraspecific genetic diversity across its distribution range within southwestern Japan (Matsui *et al*., 2006). The genetic distances found among local populations of *H. nebulosus* are often larger than those observed between this species and *H. yangi* or *H. tsuensis* (Matsui *et al*., 2006). Actually, *H. nebulosus* is a composite of four different groups, and the population treated here belongs to the western group, which includes the nominal population (*H. nebulosus* sensu stricto). Differentiation among populations of *H. nebulosus* (sensu lato) within Japan would probably have started much earlier than those among *H. nebulosus* (sensu stricto), *H. yangi*, and *H. tsuensis*.

Hynobius tsuensis is noteworthy, because despite its close genetic similarity with *H. nebulosus* (sensu stricto) and *H. yangi*, it is purely a lotic breeder with strongly modified egg sacs, unlike other two species (Sato, 1943). Although some populations of *H. nebulosus* (sensu lato) breed in streams (Matsui *et al*., 2006), the degree of adaptation to the lotic environment is much less than in *H. tsuensis*. Probably the steep, montane environments of Tsushima forced the ancestral populations to acquire a breeding habit in flowing water. Once this habit was attained, the resident population would have never interchanged genetically with lentic breeding ancestors of *H. yangi* and/or *H nebulosus* due to ecological isolation, even during several later land connections between Tsushima and the surrounding areas during glacial periods in the Pleistocene.

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